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Whole-genome comparison of two *Acinetobacter baumannii* isolates from a single patient, where resistance developed during tigecycline therapy

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Running title: Genome sequencing of *A. baumannii* from a patient treated with tigecycline

Keywords: OXA-23 clone 1; glycylcycline resistance; comparative genomics

***Objectives*:** The whole genomes of two *Acinetobacter baumannii* isolates recovered from a single patient were sequenced to gain insight into the nature and extent of genomic plasticity in this important nosocomial pathogen over the course of a short infection. The first, AB210, was recovered before tigecycline therapy and was susceptible to this agent; the second, AB211, was recovered after therapy and was resistant.

***Methods*:** DNA from AB210 was sequenced by 454 GS FLX pyrosequencing according to the standard protocol for whole-genome shotgun sequencing, producing ~250-bp fragment reads. AB211 was shotgun-sequenced using the Illumina Genetic Analyzer to produce fragment reads of exactly 36-bp. Single nucleotide polymorphisms (SNPs) and large deletions detected in AB211 in relation to AB210 were confirmed by PCR and DNA sequencing.

***Results*:** Automated gene-prediction detected 3,850 putative coding sequences (CDS). Sequence analysis demonstrated the presence of plasmids pAB0057 and pACICU2 in both isolates. Eighteen putative SNPs were detected between the pre- and post-therapy isolates, AB210 and AB211. Three contigs in AB210 were not covered by reads in AB211, representing three deletions of approximately 15, 44 and 17 kb.

***Conclusions*:** This study demonstrates that significant differences were detectable between two bacterial isolates recovered one week apart from the same patient, and reveals the potential of whole-genome sequencing as a tool for elucidating the processes responsible for changes in antibiotic susceptibility profiles.

Introduction

Acinetobacter baumannii is an important nosocomial pathogen, with multidrug-resistant (MDR) and even pan-drug-resistant strains reported world-wide.[1] In the UK, carbapenem-resistant clonal lineages limit available treatment options. One successful lineage, designated OXA-23 clone 1, belonging to European clone II, has been recovered from over 60 hospitals, clustered mainly in London and South-East England.[2] Representative isolates of this clone are usually susceptible to colistin and tigecycline only. We previously reported the emergence of tigecycline resistance during antibiotic therapy in the OXA-23 clone 1 epidemic lineage, and showed that increased expression of the resistance-nodulation-division (RND) efflux system, AdeABC was responsible for the resistance phenotype.[3]

The recent availability of rapid and inexpensive whole-genome sequencing permits detailed investigation of genetic differences between pairs of bacterial isolates. In *A. baumannii* whole-genome studies have thus far focused either on comparing distinct antibiotic-susceptible and MDR strains,[4,5] or related isolates from different patients.[6] The results of these and other similar studies[7] point to a high degree of genome plasticity, the rapid emergence of antibiotic resistance, and considerable genetic variability even among closely-related isolates.

Tigecycline is used as a treatment of last resort for MDR *A. baumannii* infection, despite a lack of formal trial data and the emergence of resistance is a major concern. We sequenced the genomes of two *A. baumannii* isolates from a single patient, the first recovered before tigecycline therapy and susceptible to this agent, the second after one week of therapy for an intra-abdominal infection and resistant. The study aimed to gain insight into the nature and extent of genomic plasticity over the course of a short infection.

Materials and Methods

Bacterial isolates

Clinical isolates AB210 and AB211 have been described previously.[3] As OXA-23 clone 1 representatives, they belong to the globally successful European clone II group, and were assigned to Group 1 by the multiplex PCR method described by Turton *et al*.[8] They were typed by PFGE of *Apa*I-digested genomic DNA (Figure 1), as described previously,[2] and the presence of $bla_{\text{OXA-23-like}}$ was confirmed by multiplex PCR.[9]

Antimicrobial susceptibility testing and DNA manipulations

MICs were determined by BSAC agar dilution or Etest (AB bioMérieux, Solna, Sweden) on IsoSensitest agar (Oxiod, Basingstoke, UK) with the results interpreted according to BSAC guidelines.[9] Genomic DNA was extracted with the Wizard Genomic DNA Purification Kit (Promega, Southampton, UK) and was used as template for DNA sequencing. Plasmids were isolated from AB210 and AB211 using the PureYield Plasmid Miniprep System (Promega) and analysed by agarose gel electrophoresis.

Whole-genome DNA sequencing and data analysis

DNA from AB210 was sequenced by 454 GS FLX pyrosequencing (Roche, Branford, Connecticut, USA) according to the standard protocol for whole-genome shotgun sequencing, producing ~250 bp fragment reads. AB211 was shotgun sequenced using the Illumina Genetic Analyzer (Illumina, Saffron Walden, UK) to produce fragment reads of exactly 36-bp. All sequencing was performed at GATC Biotech Ltd (Constance, Germany). A draft genome assembly for AB210 was produced from flowgram data, using Newbler 2.5 (Roche). The Newbler command-line option '-rip' was used to ensure reads were aligned to single contigs

only. The resulting contigs were annotated by reference to the related strain *A. baumannii* ACICU[10] (also belonging to European clone II) using the automated annotation pipeline on the xBASE server.[11]

Illumina reads for isolate AB211 were mapped against the draft AB210 assembly using Bowtie 0.12.0.[12] For the purposes of single nucleotide polymorphism (SNP) detection, Bowtie was run with parameter '-m 0' to suppress alignments that map equally to multiple locations in the genome. To detect deletions this setting was not used. A consensus pileup was produced using SAMtools,[13] and putative SNPs were called using Varscan 2.2[14] with the following parameters: minimum coverage (10), min-reads2 (2), min-avg-qual (15), min-var-freq (0.9). To detect microindels (insertion or deletion events) less than 3-bases long, AB211 reads were additionally mapped using Novoalign 2.5.[15] Whole-genome alignments were visualised and SNPs and deletions manually inspected using the output files from the above steps using BAMview. [16]

Confirmation of SNPs and chromosomal deletions

SNPs and deletions detected in AB211 in relation to AB210 were confirmed by PCR and DNA sequencing using the primers listed in Table S1. Nucleotide sequences of the resulting amplicons were determined with an ABI 3730*xl* DNA analyser (Applied Biosystems, Warrington, UK).

Results & Discussion

Antibiotic susceptibilities

MICs of tigecycline, tobramycin, amikacin, gentamicin and azithromycin for the pre-therapy isolate AB210 were 0.5, >32, >64, >32 and >256 mg/L, respectively, while MICs for the post-therapy isolate AB211 were 16, 2, 4, 8 and >256 mg/L, respectively.

Sequencing results

Sequencing produced >128 million and >156 million sequence reads for AB210 and AB211, respectively. The assembly of AB210 resulted in 91 contigs larger than 500-bp, comprising 4.06 megabases of sequence and representing a median 29-fold coverage. Automated gene-prediction detected 3,850 putative coding sequences (CDS), of which 3,504 were homologous (defined as BLASTP e-value ≤ 1e-05) to a sequence in the reference genome of *A. baumannii* ACICU. The vast majority (96.6 %) of the AB211 reads mapped to a region on the AB210 genome. The AB210 draft assembly has been deposited in GenBank (accession number: AEOX00000000) and raw sequence reads for AB210 and AB211 have been submitted to NCBI's Sequence Read Archive under Study Accession Number SRP004860.

Plasmid profile

Plasmid profiles of AB210 and AB211 were identical and showed the presence of two plasmids in each isolate (data not shown). Sequence analysis demonstrated the presence of a 9-kb contig in AB210 which displayed 99.98 % identity to the previously characterised pAB0057 plasmid.[5] This was seen at high sequence read coverage in both AB210 and AB211, suggesting it was present as multiple copies. Three other contigs, totalling 65 kb, were seen at below-average

coverage; taken together these were a full match in length and nucleotide identity to the complete pACICU2 plasmid.[10]

AB210 virulence genes and resistance islands

Resistance islands (RIs) have been detected in all sequenced *A. baumannii* genomes containing multiple resistance determinants. They are composite transposons that are complex in nature and which have been designated AbaR (*A. baumannii* resistance).[4] They share a common insertion site (*comM*) but vary considerably among isolates in terms of the exact genetic composition, with that from ACICU, a representative of European clone II being considerably reduced in size compared to those found in representatives of European clone I.[10,17] Clinical isolates AB210 and AB211 were found to contain an AbaR-type RI. In the former isolate (GenBank accession number HQ700358) this was shown to contain sequence corresponding to nucleotides 587330-599047 of strain AB0057 (GenBank accession number CP001182), with a 2.85 kb section absent; this is an AbaR4-type island, and contains $bla_{\text{OXA-23}}$.

SNPs between AB210 and AB211

Eighteen putative SNPs were detected between the pre- and post-therapy isolates. Only one of these was located outside of coding regions at -35 bp upstream of *ureJ* which encodes a hydrogenase/urease accessory protein (AB210 locus tag: AB210-1_2203). The location of this SNP suggests the possibility of regulatory significance although *ureJ* appears to be part of a urease gene cluster which is co-transcribed as an operon in other species.[18] Of the remaining 17, eight were synonymous mutations whereas nine were non-synonymous including one missense mutation (Table 1). Seventeen (94 %) of the SNPs were transitions. Eight of the nine non-

synonymous SNPs could be confirmed by PCR and sequencing while one was not validated (Table 1 and Table S1). Several of these were located within genes predicted to be involved in core biological functions, including translation (*dusB*), nucleic acid biosynthesis, α-ketoglutarate and arabinose transport, environmental sensing (the signal transduction histidine kinase gene, *adeS* which had previously been identified through a candidate-gene approach[3]), and signalling. The mutation in *adeS* is believed to be responsible for up-regulation of the AdeABC efflux system and hence tigecycline resistance. Two SNPs were located within a gene coding for a GGDEF domain-containing protein, one of which was a non-synonymous mutation whilst the other introduced an internal stop codon, thus giving rise to a truncated product (Table 1). These proteins are enzymes that catalyze the synthesis of cyclic-di-GMP, which has been recognized recently as an important second messenger in bacteria and is implicated in adhesin and extrapolysaccharide biosynthesis.[19]

Large structural changes in the genomes of AB210 and AB211

Three contigs in AB210 were not covered by reads in AB211, these putative deletions were designated ROD1, 2 and 3. The first, ROD1, was approximately 15 kb in length. This deletion disrupted the coding sequence of the DNA mismatch repair gene *mutS* (AB210-1_2445) by eliminating the N-terminal *mutS-I* domain. Aside from encoding this mismatch recognition enzyme, ROD1 also encoded a DMT superfamily permease (AB210-1_2447) and an MFS permease (AB210-1_2451), transcriptional regulators (AB210-1_2450; AB210-1_2453), an EAL domain-containing protein (AB210-1_2448), responsible for the degradation of cyclic-di-GMP.[19] At approximately 44 kb ROD2 was the largest deleted region and comprised of genes encoding for transcriptional regulators (AB210-1_3253; AB210-1_3262; AB210-1_3269; AB210-1_3273), ion channels and transporters (AB210-1_3254; AB210-1_3259; [AB210-1_3275;

AB210-1_3276; AB210-1_3277]), a class A β-lactamase enzyme (AB210-1_3248) and components of a type VI secretion system (AB210-1_3280; AB210-1_3281).[20] Interestingly, part of the type VI secretion locus was missing even in AB210, suggesting that this was a degenerate system in both isolates. ROD1 and ROD2 are contiguous in *A. baumannii* ACICU, suggesting this may be a single deletion, but this could not be confirmed experimentally for AB210 by PCR (data not shown). ROD3, approximately 17 kb in length, included a class 1 integron containing antibiotic resistance genes including macrolide resistance determinants (AB210-1_3691 [phosphotransferase]; AB210-1_3692 [an efflux protein]) and several genes encoding aminoglycoside resistance determinants, namely *aac*(*6'*)-*Ib* (AB210-1_3701), two copies of *aadA* (AB210-1_3699; AB210-1_3700) and *armA* (AB210-1_3695), which encodes a 16S rRNA methylase.

Implications for Acinetobacter *evolution*

The extent of genomic changes detected here are consistent with the marked changes in phenotype, particularly the loss of aminoglycoside resistance in AB211. However, we were unable to determine whether these changes were the result of rapid evolution during the course of infection and treatment, or whether the patient initially had a mixed infection (or re-infection), involving different variants of the same defined clone, with subsequent selection for tigecycline resistance.

The disruption of *mutS*, an important DNA mismatch repair gene, is significant and suggests the possibility of a hypermutator phenotype, which may have contributed to the relatively large number of SNPs. Previous work in *Acinetobacter* sp. ADP1 has shown that *mutS*

196 preferentially recognises and repairs transitions,[21] so its disruption in AB211 is consistent with
197 our observation that 94 % of the SNPs belonged to this class.

198 The absence of ROD3 is consistent with the change in aminoglycoside resistance
199 between AB210 and AB211, with MICs of tobramycin, amikacin and gentamicin reduced at
200 least 8-fold in AB211. It is notable that the development of tigecycline resistance was
201 accompanied by increased susceptibility to other antibiotics through a large genomic deletion.

202 GGDEF and EAL-containing proteins have been implicated in sessile to planktonic
203 shifts. Taken together, the termination in a GGDEF domain-containing protein as well as the loss
204 of an EAL-domain containing protein in ROD1 may be advantageous during the process of
205 infection though this remains to experimentally determined.

206 In this study, whole-genome sequencing gave insight into the nature of genetic changes
207 between isolates under selection pressure through antibiotic therapy and a hostile host
208 environment. This study has demonstrated significant differences between two *A. baumannii*
209 isolates belonging to the same epidemic lineage, collected one week apart from the same patient.
210 Such studies are able to shed light on the relative importance of SNPs and transposon
211 mutagenesis on the evolution of *A. baumannii* and can generate hypotheses into the nature of
212 antibiotic resistance and virulence. Although further studies are needed to assess the extent of
213 genetic diversity among populations of *A. baumannii* in a single patient, we clearly demonstrated
214 the potential of whole-genome sequencing as an important tool for helping elucidate the
215 evolutionary processes responsible for the rapid development of antibiotic resistance in this
216 important nosocomial pathogen.

217

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Transparency Declarations

D. M. L. has (i) received research grants from Wyeth and Pfizer, (ii) spoken at meetings organised by Wyeth and Pfizer, (iii) received sponsorship to travel to congresses from Wyeth and Pfizer, as well as from numerous other pharmaceutical and diagnostic companies. He holds shares in GlaxoSmithKline, Merck, AstraZeneca, Dechra and Pfizer; he acts also as Enduring Attorney for a close relative, managing further holdings in GlaxoSmithKline and EcoAnimal Health. N. W. has received research grants from Wyeth. M. E., M. D., J. F. T., A. U., T. G., D. M. L. and N. W. are employees of the HPA and are influenced by its views on antibiotic use and prescribing. M. H., D. W. W. and C. P. T. have received sponsorship to attend conferences from Wyeth. N. L. and M. J. P. : none to declare.

References

1. Towner KJ. *Acinetobacter*: an old friend, but a new enemy. *J Hosp Infect* 2009; **73**: 355-63.

2. Coelho JM, Turton JF, Kaufmann ME, *et al*. Occurrence of carbapenem-resistant *Acinetobacter baumannii* clones at multiple hospitals in London and Southeast England. *J Clin Microbiol* 2006; **44**: 3623-7.

3. Hornsey M, Ellington MJ, Doumith M, *et al*. AdeABC-mediated efflux and tigecycline MICs for epidemic clones of *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy* 2010; **65**: 1589-93.

4. Fournier PE, Vallenet D, Barbe V, *et al*. Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. *PLoS Genet* 2006; **2**: e7.

5. Adams MD, Goglin K, Molyneaux N, *et al*. Comparative genome sequence analysis of multidrug-resistant *Acinetobacter baumannii*. *J Bacteriol* 2008; **190**: 8053-64.

6. Adams MD, Chan ER, Molyneaux ND, *et al*. Genomewide analysis of divergence of antibiotic resistance determinants in closely related isolates of *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2010; **54**: 3569-77.

7. Adams MD, Nickel GC, Bajaksouzian S, *et al*. Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. *Antimicrobial Agents and Chemotherapy* 2009; **53**: 3628-34.

8. Turton JF, Gabriel SN, Valderrey C, *et al*. Use of sequence-based typing and multiplex PCR to identify clonal lineages of outbreak strains of *Acinetobacter baumannii*. *Clin Microbiol Infect* 2007; **13**: 807-15.

9. Andrews JM. BSAC standardized disc susceptibility testing method. Version 9.1, March 2010. http://www.bsac.org.uk/Resources/BSAC/Version_9.1_March_2010_final.pdf (22 March 2011, date last accessed).

10. Iacono M, Villa L, Fortini D, *et al*. Whole-genome pyrosequencing of an epidemic multidrug-resistant *Acinetobacter baumannii* strain belonging to the European clone II group. *Antimicrobial Agents and Chemotherapy* 2008; **52**: 2616-25.

11. Chaudhuri RR, Loman NJ, Snyder LA, *et al*. xBASE2: a comprehensive resource for comparative bacterial genomics. *Nucleic Acids Res* 2008; **36**: D543-D546.

12. Langmead B, Trapnell C, Pop M, *et al*. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* 2009; **10**: R25.

13. Li H, Handsaker B, Wysoker A, *et al*. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 2009; **25**: 2078-9.

14. Koboldt DC, Chen K, Wylie T, *et al*. VarScan: variant detection in massively parallel sequencing of individual and pooled samples. *Bioinformatics* 2009; **25**: 2283-5.

15. Krawitz P, Rodelsperger C, Jager M, *et al*. Microindel detection in short-read sequence data. *Bioinformatics* 2010; **26**: 722-9.

16. Carver T, Bohme U, Otto TD, *et al*. BamView: viewing mapped read alignment data in the context of the reference sequence. *Bioinformatics* 2010; **26**: 676-7.

17. Post V, White PA, Hall RM. Evolution of AbaR-type genomic resistance islands in multiply antibiotic-resistant *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy* 2010; **65**: 1162-70.

18. McMillan DJ, Mau M, Walker MJ. Characterisation of the urease gene cluster in *Bordetella bronchiseptica*. *Gene* 1998; **208**: 243-51.

19. Hengge R. Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* 2009; **7**: 263-73.

20. Bingle LE, Bailey CM, Pallen MJ. Type VI secretion: a beginner's guide. *Curr Opin Microbiol* 2008; **11**: 3-8.

21. Young DM, Ornston LN. Functions of the mismatch repair gene *mutS* from *Acinetobacter* sp. strain ADP1. *J Bacteriol* 2001; **183**: 6822-31.

Table 1. Confirmed SNPs indentified in clinical isolate AB211 resulting in amino acid substitution or termin

SNP	Position in AB210 assembly	Locus tag in AB210 assembly	Protein product	Amino acid identity	
				AB210	AB211
1	159509	AB210-1_0138	tRNA-dihydrouridine synthase, DusB	A	T
2	639321	AB210-1_0587	nucleoside-diphosphate-sugar epimerase	T	A
3	755474	AB210-1_0703	major facilitator superfamily permease	V	A
4	1469178	AB210-1_1405	hypothetical protein	A	V
5	2548057	AB210-1_2423	major facilitator superfamily permease	A	T
6	2852737	AB210-1_2721	Signal transduction histidine kinase, AdeS	A	V
7	3362158	AB210-1_3207	GGDEF domain-containing protein	Q	*
8	3362175	AB210-1_3207	GGDEF domain-containing protein	G	V

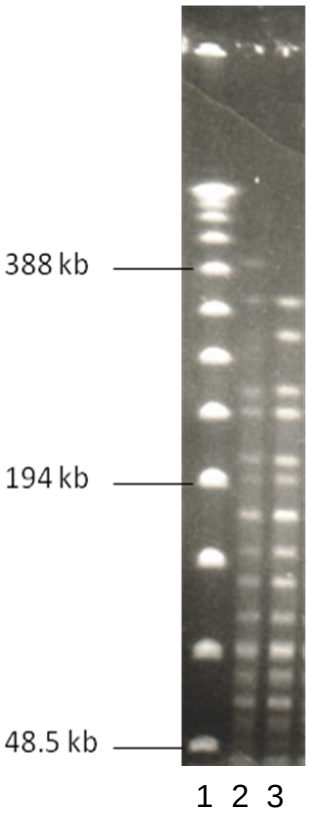

Figure 1.

Figure Legands

Figure 1. PFGE profiles of AB210 (lane 2) and AB211 (lane 3).